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Absence of Mutagenicity in the Urine of Autopsy Service Workers Exposed to Formaldehyde: Factors Influencing Mutagenicity Testing of Urine

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16. ABSTRACT

This study examined the effect of exposure to autopsy workers to formaldehyde using urinary mutagenicity testing with S. typhimurium. A matched control group was also studied. Additional studies including the recovery of histidine from urine samples, the determination of histidine in urine samples and the effect of exogenous and urinary histidine on the mutagenicity assay were also carried out. Results showed that there was no increase in mutagenicity in the autopsy workers as compared to the control group. A large proportion of the exposed individuals had toxic urine while only two of the control group has similar toxic urine. However, the toxic material in the urine samples has been isolated and does not appear to be related to the formaldehyde exposure. In conclusion, exogenous histidine and certain growth factors present in human urine can affect the outcome of both plate incorporation and fructuation assays for mutagenicity and care must be taken when interpreting results of this type of mutagenicity studies.

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Absence of mutagenicity in the urine of autopsy service workers exposed to formaldehyde: factors influencing mutagenicity testing of urine

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Summary. Hospital autopsy service workers and a matched control group were studied using a battery of genetic monitoring tests performed on samples of blood, semen and urine. The results of the analysis of urine for mutagens are described in this report. The participants in the study were matched with the controls for sex, age and their use of alcohol, tobacco and marijuana. Information was collected on general health, usage of medications and any exposure which might affect the outcome of the study. Individuals were sampled three times at approximately two month intervals. Time weighed average exposures to formaldehyde in the work areas were estimated at 0.61 to 1.32 ppm. Additionally, studies were carried out which examined various parameters affecting the testing of human urine samples for mutagenicity.

No increase in mutagenicity was seen in the autopsy workers as compared to the control group. One individual who was receiving metronidazole and one control who smoked two packs of cigarettes per day had significantly mutagenic urine. A large proportion of the exposed individuals had toxic urine while only two of the control individuals had similar toxic urine. The material responsible for the toxicity has been isolated and purified but does not appear to be related to the formaldehyde exposure.

Studies on the parameters affecting mutagenicity testing of urine with *Salmonella typhimurium* suggest that in the plate incorporation assay, TA100, but not TA98, can be affected by exogenous histidine. Furthermore, with the conditions employed in this study, 3 to 4% of labeled histidine added to urine samples was retained by the XAD-2 and subsequently eluted in the urine concentrate. Urinary histidine levels of unconcentrated samples ranged from 112 to 2614 nmol per ml (mean 994 nmol per ml) and the amount of histidine present correlated with the corresponding increases in histidine revertants with strain TA100.

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Key words: Formaldehyde – Autopsy personnel – Mutagenicity – Urine – Histidine

Introduction

With the findings that formaldehyde is a carcinogen in rats at relatively low doses (Swenberg et al. 1980; Albert et al. 1982), there has been growing concern over both the general public's exposure to low levels of formaldehyde and certain occupational settings where exposure may be considerably higher (Blackwell et al. 1981; Niemela and Vainio 1981). Nonoccupational exposures result primarily from offgassing of formaldehyde by textiles and certain building products, especially foam insulation and particle board. Occupational exposures are the result of manufacturing of formaldehyde-based products, industrial processes involving the use of formaldehyde and exposure of autopsy and pathology workers to formalin (IARC 1983). A study by Fleig et al. (1982) involving a group of workers in formaldehyde manufacturing and processing failed to demonstrate any increase in chromosomal aberrations even though the mean time worked was 28 years. At the present time, epidemiological data are not sufficient to assess the carcinogenicity of formaldehyde in humans (IARC 1983). Until the present study was undertaken, one primary occupational exposure to relatively high levels of formaldehyde, autopsy and pathology personnel (Blackwell et al. 1981), had not been examined for possible genotoxic damage. Our comprehensive study took into account the daily and intermittent levels to which a group of autopsy workers was exposed, medical history of the study participants and other factors which could affect the outcomes of the various tests (Ward et al. 1983). The genetic end points which were evaluated included the analysis of peripheral lymphocytes for sister chromatid exchanges, chromosomal aberrations and DNA damage; the analysis of semen for sperm count, abnormal morphology and 2F-body frequencies; the analysis of globin for formaldehyde adducts; and the analysis of the urine for mutagenic substances. The results of the urine analysis are described in this report along with several factors which may affect the outcome of mutagenicity testing of human urine with *S. typhimurium*. Results of the sperm evaluation have been published elsewhere (Ward et al. 1984).

Materials and methods

Population recruitment, evaluation and selection

Subjects for the exposed group were recruited from the staff of the Autopsy Service and the Pathology Department of the University of Texas Medical Branch at Galveston and those for the control group from the faculty, staff and students of the medical school.

Participants were asked to complete a questionnaire which inquired about age, sex, occupation, potential sources of occupational exposure to toxic substances, customary use of tobacco, alcohol and recreational drugs (marijuana), history of major illnesses and current and past use of medications and reported exposure to organic solvents, pesticides and X-irradiation. Each respondent in the exposed group was matched with a respondent in the non-

exposed group. Criteria for matching were (in order of importance) sex, age (to within five years), and use of tobacco, alcohol and recreational drugs. Health, medications and incidental exposures were not used as a basis for matching but were considered in selecting subjects for study and evaluating results. Most subjects were selected at the beginning of the study but two pathology residents rotating through the autopsy service and a newly hired technician were recruited and matched with controls selected from the pool of original respondents. The majority of the exposed population had worked on the autopsy service for at least six months prior to the study. The study concluded with nineteen individuals in the exposed group and twenty in the control group including one individual receiving metronidazole. The studies on the effect of urinary histidine levels on revertants were performed with urine samples collected from an additional group of individuals not included in the autopsy study.

Sample collection and processing

Urine samples were collected from individuals in pre-coded plastic specimen containers. Approximately 150–200 ml of urine was collected between noon and 3:00 pm on Monday or Thursday. Two exposed individuals and two matched controls were sampled on each of these days. The pH and specific gravity of each sample were determined after which the samples were centrifuged at 1500 rpm for 10 min. The samples were then filter-sterilized with a 0.45 um filter and a prefilter. Next, 100 ml of each sample was incubated with 1000 units of β-glucuronidase (Type IX Sigma Chemical Co., St. Louis, MO, USA) at 37°C, with shaking, for 16 h.

After incubation the urine samples were extracted using the method of Yamasaki and Ames (1977) with some modifications. Amberlite XAD-2 resin (Mallinckrodt Chemical Works, St. Louis, MO, USA) was washed repeatedly with distilled-deionized water, acetone and again with water. It was added to 10 mm I.D. × 30 cm L. glass columns with Teflon stopcocks to a bed height of 20 cm. After packing, the columns were washed with 50–100 ml distilled-deionized water. The urine samples were then passed through the columns at a rate of 1–2 ml per min. When the entire sample had passed through the resin, the columns were flushed with nitrogen, washed with 1.0 ml distilled-deionized water and again flushed with nitrogen. The material remaining on the resin was then eluted with 10 ml acetone which was reduced to 0.5 ml at 50°C under a stream of nitrogen. Finally, the volume was adjusted to 2.0 ml with dimethyl sulfoxide (DMSO) and mixed well to resuspend the concentrate. The samples were usually tested immediately after concentration or stored at −80°C for less than a week before testing.

Mutagenicity assay

The mutagenicity assay was carried out according to Ames et al. (1975) with slight modifications. These included the use of Spizizen's minimal medium (Spizizen 1958) in place of Vogel-Bonner medium and the addition of the biotin and histidine (0.25 and 2.0 μg per ml, final concentration) to the minimal medium rather than to the top agar. Strains TA100 and TA98 of *S. typhimurium* were supplied by B.N. Ames, Berkeley, CA, USA. The plates received either 0.5 ml of S9 mix prepared from Aroclor 1254-induced Sprague-Dawley rats or 0.5 ml of buffer. All urine extracts were tested in duplicate at 50 and 100 μl per plate, with and without S9 using both strains of bacteria. Unconcentrated, filter-sterilized urine from cyclophosphamide treated rats (50 mg per kg, i.p., Mead-Johnson, Evansville, IN, USA) and 2-aminoanthracene, 5 μg per plate, Aldrich Chemical Co., Milwaukee, WI, USA) were used as positive controls. Sterility controls were run on all urine extracts, the S9 mix and the buffer. The plates were incubated for 48 h at 37°C in the dark after which they were counted on an automatic colony counter. All sample preparations and mutagenicity assays were carried out at ambient temperature under yellow lighting.

Effects of histidine on the plate incorporation assay

In order to quantitate the efficiency of the urine extraction procedure in removing urinary histidine, a pooled sample from several control individuals not included in the above study

was divided into two 50 ml fractions and 2.0 μCi of ^{14}C-histidine (specific activity, 312 mCi/mmol, New England Nuclear, Boston, MA, USA) was added to each. After mixing, aliquots were removed for liquid scintillation counting and each fraction was applied to an XAD-2 column as described above. One of the columns was washed with water after which both were eluted with 10 ml acetone. Aliquots were again counted and the per cent recovery calculated.

The effect of exogenous histidine on the plate incorporation assay was determined by adding known amounts of histidine to the top agar and plating with TA100 and TA98 as described above for the mutagenicity assays. In addition, the histidine levels in the unconcentrated urine of nine additional control individuals was measured by amino acid analysis and the number of revertants produced by 200 μl of each sample was compared to the histidine levels found.

Results

Study population

Table 1 summarizes the characteristics of the exposed and the nonexposed study populations. The one factor which would affect the outcome of the

Table 1. Population description for personnel in autopsy study

	Experimental group	
	Exposed	Non-exposed
Sex		
Male	15	15
Female	4	5
	19	20
Age		
<30	8	11
30–40	6	4
41–50	2	2
>50	3	3
Cigarettes/day		
0	15	14
1–19	2	2
≥20	1	2
Pipe or cigars/day	2	2
Alcoholic drinks/day		
0	5	4
<1	4	9
1–4	10	7
Cups coffee/day		
0	3	4
1–3	3	11
>3	13	5

Table 2. Mutagenicity of urine samples of non-exposed and formaldehyde-exposed individuals

Code	Number	Smoker[b]	Net revertants:/plate[a]											
			Sample I				Sample II				Sample III			
			Strain S9											
			TA100		TA98		TA100		TA98		TA100		TA98	
			−	+	−	+	−	+	−	+	−	+	−	+
Non-exposed group														
1.	018	N	67	33	14	20	—	—	0	7	32	74	2	19
2.	016	N	8	13	5	26	12	34	6	16	0	51	6	19
3.	077	N	—	39	—	5	0	58	1	5	6	22	0	5
4.	087	N	21	15	7	9	39	79	6	4	0	39	7	23
5.	049	N	0	35	10	8	—	—	—	—	—	—	—	—
6.	132	N	3	25	5	11	0	32	0	11	T	26	2	T
7.	085	N	T	114	T	0	0	18	6	0	—	—	—	—
8.	113	N	T	2	T	7	T	T	T	T	T	T	T	T
9.	103	N	0	33	5	18	5	45	1	12	—	—	—	—
10.	119	N	12	18	0	0	15	50	0	10	7	46	2	20
11.	008[c]	S	29	18	10	17	0	10	10	28	T	T	T	T
12.	118	N	—	10	—	14	13	6	0	5	8	39	11	12
13.	094	N	5	27	0	15	6	19	0	30	30	11	0	0
14.	117	N	—	18	—	12	18	40	0	21	25	56	4	9
15.	014	N	23	37	8	1	0	40	0	14	0	36	7	6
16.	110	S	48	70	16	215[d]	2	113	4	222	38	128	14	188
17.	121	N	0	0	0	8	5	22	2	4	0	51	0	18
18.	046	N	33	37	9	13	14	44	0	8	30	57	8	20
19.	054	N	13	6	5	13	7	46	1	0	12	27	5	5
20.	074[e]	S	3000+	3000+	37	103	37	40	14	24	—	—	—	—

Table 2 (continued)

Code	Number	Smoker[b]	Net revertants/plate[a]											
			Sample I				Sample II				Sample III			
			Strain S9											
			TA100		TA98		TA100		TA98		TA100		TA98	
			−	+	−	+	−	+	−	+	−	+	−	+
Exposured group														
1.	058	N	T	40	T	5	0	26	0	7	T	T	T	T
2.	062	N	T	0	T	10	T	T	T	T	T	0	T	12
3.	066	S	0	31	2	22	1	32	6	16	0	38	0	30
4.	096	S	T	43	0	10	T	T	T	T	0	47	7	8
5.	130	N	32	52	11	1	—	—	—	—	—	—	—	—
6.	129	N	0	7	1	8	—	—	—	—	—	—	—	—
7.	131	N	0	73	8	7	T	T	3	T	—	—	—	—
8.	057	N	2	36	6	13	17	71	4	12	0	2	8	12
9.	060	N	0	24	7	29	0	18	0	18	15	0	0	4
10.	097	N	8	5	6	0	0	40	0	14	T	72	14	5
11.	064	S	2	0	4	21	0	14	0	36	0	52	0	47
12.	100	N	T	T	T	T	T	T	T	T	T	T	T	T
13.	068	N	9	22	2	18	18	47	1	3	T	37	T	3
14.	098	N	—	28	—	27	14	29	T	21	14	69	0	14
15.	061	N	14	39	0	20	—	—	—	—	—	—	—	—
16.	065	S	37	0	3	32	18	27	0	30	12	61	7	30
17.	059[f]	N	T	T	T	T	T	29	T	0	T	T	T	T
18.	067	N	8	7	0	4	37	47	3	7	0	36	3	17
19.	069	N	T	25	T	4	0	41	0	0	32	41	0	0

[a] Mean of treated plates minus the mean of control plates. Only the higher value for either 50 or 100 μl is reported. Mean ± SD (n = 35) for control plates were: TA100 (−S9) 149.1 ± 20.3; TA100 (+S9) 162.2 ± 21.0; TA98 (−S9) 28.6 ± 7.8; TA98 (+S9) 46.0 ± 8.8. Mean ± SD for mutagen treated plates were: 100 μl urine from cyclophosphamide treated rats, TA100 (−S9) 150.7 ± 56.6; TA100 (+S9) 168.5 ± 51.3 and 5 μg 2-amino anthracene, TA100 (+S9) 1500–2500; TA98 (+S9) 1500–2500

0 = Less than or equal to control plates but with no reduction in background lawn
T = Partial or complete absence of background lawn
— = No sample available

[b] N = Nonsmoker
S = Smoker
096 minimal cigar/pipe smoker

[c] 008 III toxicity due to drug exposure

[d] Underlined values are greater than 2 times their concurrent control

[e] 074 I is day 6 of metronidozole therapy

[f] Toxicity due to multiple drug therapy

mutagenicity assay the greatest, cigarette smoking, was limited to three heavy smokers (i.e. greater than a pack per day).

Formaldehyde exposure

During the time of the sampling, formaldehyde levels were measured in the autopsy service areas using the chromotropic acid method (NIOSH 1977), the results of which have been reported elsewhere (Coldiron et al. 1983). Using both area and personal breathing zone monitoring, it was seen that formaldehyde levels ranged from 0.1 ppm (detection limit) for time spent outside the immediate work area to a maximum of 5.8 ppm for specific activities. Time weighted average exposures were estimated to be between 0.61 and 1.32 ppm.

Mutagenicity of urine concentrates

When all urine concentrates were tested at 50 and 100 µl per plate (i.e. an equivalent of 2.5 and 5.0 ml urine per plate, respectively), with and without S9, the only samples which demonstrated any substantial levels of mutagenicity were those from the individual sampled while receiving metronidazole (day six of therapy) and all three samples from one of the controls (110) who smoked two packs of cigarettes per day (Table 2). The samples from No. 110 were shown to contain a mutagenic metabolite of 2-naphthylamine (Connor et al. 1983a). Other than these four, most samples produced little to no increase in revertants (i.e. greater than two times the background) with either strain. However, there were slight mutagenic responses observed with two or three of the cigarette smokers when assayed with S9 and TA98.

The most outstanding observation with the urine extracts was the finding of a significant number of toxic samples in the exposed group (13) as compared to only four in the control group (Table 3). Also, an individual was more likely to have more than one of the three samples toxic if any were found to be toxic. With the toxic samples, it was seen that the addition of the S9 reduced the toxicity and that when the toxic samples were tested at lower concentrations, no mutagenicity was observed (data not shown). Subsequent analysis of the toxic samples determined that the majority of them contained a common material

Table 3. Distribution of individuals with mutagenic or toxic urine samples

Experimental group	Non-mutagenic/ Non-toxic	Mutagenic	Toxic	Totals
Non-exposed	16 (42)[a]	1[b] (3)[c]	2 (4)[d]	19 (49)
Exposed	11 (27)	2 (5)[c]	5[e] (13)	18 (45)
Totals	27 (69)	3 (8)	8 (17)	37 (94)

[a] Number in parentheses is the total number of samples in each category
[b] 074 not included due to metronidazole therapy
[c] All mutagenic samples from cigarette smokers
[d] 008 III not included due to drug therapy
[e] 059 not included due to multiple drug therapy

Table 4. Removal of ^{14}C-histidine from human urine using XAD-2

	Not washed	Washed
Total DPM in original sample (50 ml)	3.68×10^6	3.68×10^6
Total DPM in concentrated urine (1 ml)	1.23×10^5	1.35×10^5
% DPM in concentrated urine (1 ml)	3.35	3.65
Total DPM in aqueous effluent (52 ml)	3.51×10^6	3.39×10^6
% DPM in aqueous effluent (52 ml)	95.38	91.99
% DPM recovered	98.73	95.65

Table 5. Histidine levels in human urine and resulting mutagenicity towards *S. typhimurium* TA100

Sample No.	nmol histidine per ml	Net revertants per 200 µl[a]
1113[b]	111.9	0
1109[b]	117.5	11
1111	128.3	T[c]
1114	398.2	T
1106	729.7	92
1115	937.3	66
1110	1162.2	171
1112	1688.0	132
1108	1944.9	135
1107	2614.4	219
Mean	994.2	

[a] Spontaneous background for TA100 was 164 revertants per plate
[b] Both samples from same individual
[c] Toxic

which was excreted as a conjugate of glucuronic acid and was toxic to TA100 and TA98 (Connor et al. unpublished work).

The effects of histidine on the plate incorporation assay

It was seen that 3–4% of the ^{14}C-histidine which was added to the XAD-2 column was retained in the urine concentrate and that the water wash had little effect on removing the histidine remaining on the column (Table 4). It was also demonstrated that strain TA100 was sensitive to histidine added to the top agar while TA98 was not. However, at 100 and 60 µg per plate, TA100 and TA98, respectively, the plates exibited confluent growth and could not be counted.

When human urinary histidine levels were determined for the nine control individuals, the amount of histidine ranged from 112 to 2614 nmol per ml (Table 5). Although the inconcentrated samples were tested with several strains of *S. typhimurium,* only TA100 responded to the addition of human urine. The urinary levels of histidine and their effect on TA100 are shown in Table 5.

Discussion

The analysis of body fluids to screen workers occupationally exposed to chemical mutagens has been employed in a variety of industrial settings (Falck et al. 1979, 1980; Moller and Dybing 1980; Dolara et al. 1981; Laires et al. 1982; Nguyen et al. 1982; Sorsa et al. 1982; Heussner et al. 1985). In addition, urine from individuals receiving cancer chemotherapy drugs (Minnich et al. 1976), certain other mutagenic drugs (Connor et al. 1975; Connor et al. 1977; Roxe et al. 1980; Wheeler et al. 1981) and the urine of cigarette smokers (Yamasaki and Ames 1977) has been shown to contain excreted mutagens. These assays usually make use of the Ames *Salmonella* assay as either a plate incorporation test or as a fluctuation test. While there may be some inherent problems with such mutagenicity assays when urine concentrates are involved (Gibson et al. 1983; Venitt and Bosworth 1983; Venitt et al. 1984), we feel that with the proper controls and careful analysis of the results that this assay, coupled with other assays which may indicate exposure to genotoxins, such studies can be helpful when evaluating human exposure to chemical mutagens.

The present study examined the effect of exposure of autopsy workers to levels of formaldehyde which were between 0.61 and 1.32 ppm (TWA) with a maximum concentration of 5.8 ppm. These levels of formaldehyde have been shown to produce irritation of the eyes and mucous membranes of sensitive individuals (Loomis 1979) while 15 ppm formaldehyde exposures produced nasal tumors in rats (Swenberg et al. 1980; Albert et al. 1982). Although earlier studies have reported that formaldehyde was not mutagenic in the *Salmonella* assay, we (Connor et al. 1983b) and others (Donovan et al. 1983; Haworth et al. 1983) have recently shown that strain TA100 is mutated to some extent by formaldehyde. However, from the analysis of the mutagenicity data, it is unlikely that a mutagenic form of formaldehyde is appearing in the urine of the autopsy workers. Because formaldehyde is highly reactive with nucleophilic sites in both proteins and nucleic acids and is rapidly metabolized, little of the parent compound would be expected to be present in the urine of exposed individuals. Also, in humans it is known that much of the formaldehyde is excreted in the urine as sodium formate (IARC 1983). However, it has also been demonstrated that some of the degradation products (Feldman and Balabanova 1978) or reaction products (Auerbach et al. 1977; Fontignie-Houbrechts et al. 1982) of formaldehyde may be more toxic than formaldehyde itself. If any of these products were excreted in the urine of the autopsy workers, it was not mutagenic to either strain of *Salmonella*.

Although a significant number of the urine samples from the exposed group were toxic to the bacteria, what appears to be a similar toxin has been found in two other study groups. Analysis of the samples tested by Heussner et al. (1985) indicated that this toxin was present in the urine of a group of aluminium plant workers and also in the control group as well. Cloak and Connor (1984) found the toxin to be randomally distributed among nursing personnel whether or not they were exposed to antineoplastic drugs. Further studies indicate that the toxins from the three study populations appear to be identical (Connor et al., unpublished work).

The range of the histidine found in control individuals was between 111.5 and 2614.2 nmol per ml with a mean of 994.2 nmol per ml. In a study by Nakamura and Pisano (1976), in which they compared two fluorescent techniques for the determination of histidine in human urine, the mean values for the two techniques were 945.0 and 913.8 nmol per ml for samples from eight male subjects. The histidine values from the present study were not as uniform as those from reported by Nakamura and Pisano but the mean value was within the range reported in their study.

From the results of the histidine studies using the XAD-2, it was seen that only 3–4% of the labeled histidine was recovered in the urine concentrate. However, this appears to be sufficient, in some cases, to produce an apparent weak positive mutagenic response in TA100. Additionally, there appear to be some growth factors available in human urine which produce even higher mutagenic responses in TA100 than histidine alone. Our results are somewhat dissimilar to those of Gibson et al. (1983) who reported that TA98 was more sensitive to the urinary growth factors but not to histidine itself. Venitt and Bosworth (1983) and Venitt et al. (1984) using a fluctuation assay, concluded that this assay was sensitive to growth enhancers present in human urine which may lead to spurious results when performing mutagenicity assays. Aeshbacher et al. (1983) have reported that both histidine and certain dipeptides are able to affect the number of revertants in the plate incorporation assay. They also demonstrated that TA100 was more sensitive to histidine than TA98, TA1535, TA1537 or TA1538. Consequently, it must be concluded that histidine and certain growth factors present in human urine can influence the outcome of both the plate incorporation and fluctuation assays for mutagenicity. Even with the use of the XAD-2 column to remove most of the histidine, it is possible that residual histidine or other growth factors are capable of producing weak mutagenic responses when testing human urine concentrates. These responses are in the range of two and possibly three times the spontaneous background with the plate incorporation assay and care must be taken when interpreting results of mutagenicity studies of this type.

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